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Photoprotective herbal extract loaded nanovesicular creams inhibiting ultraviolet radiations induced photoaging

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Abstract

Botanical photochemopreventives are used to fight against the adverse effects of ultra violet rays on skin which leads to photoaging. Phytoconstituents present in *Punica granatum* extract have antioxidant, anti-carcinogenic and photoprotective properties which was proved by estimation of phenolic content, sun protection factor and antioxidant effect determination of the alcoholic extract. Extract loaded nanotransfersomes were prepared, evaluated for size, entrapment efficiency and incorporated into the cream. The presence of vesicles in the cream was confirmed by the transmission electron microscopy and the efficacy of the formulated creams by the physicochemical parameters. The nanotransfersomal and conventional cream formulations were *in vivo* tested for change in skin hydration (Corneometer), viscoelasticity (Cutometer) and sebum content (Sebumeter) . The photoprotective effect of formulations was further confirmed by biochemical studies. Improved skin properties (viscoelasticity, skin hydration, sebum content) and biochemical parameters (superoxide dismutase, catalase, total protein and ascorbic acid level) with decreased malondialdehyde level was observed with nanotransfersomal cream formulations as compared to conventional creams. The efficacy was in the order nanotransfersomal creams > Conventional creams> Empty nanotransfersomal cream > Base cream.

Keywords: Herbal nanovesicles; ellagic acid; photo protective creams; photoaging; nanotransfersomes

Introduction

The term “photoaging” was first coined in 1986 and describes the effects of chronic ultraviolet (UV) light exposure on skin [1]. However, unlike chronological aging, which depends on the passage of time, photoaging depends primarily on the degree of sun exposure and skin pigment. Photoaging could be prevented by blocking or inhibiting the UV penetration into skin, by inhibition of inflammation by antioxidants and anti-inflammatory molecules and medically based rejuvenation treatments of photoaged skin.

Several polyphenolic antioxidants of plant, such as green tea, grape seed, turmeric, pomegranate and others, have been shown to be effective *in vitro* for prevention of cellular photodamage, and skin cancer [2,3] . The use of sunscreen from childhood is recommended to prevent acute severe sunburn and to reduce the level of accumulated DNA damage caused by daily repeated exposures, and to both retard the onset of visible photoaging, and reduce the risk for melanoma and non-melanoma skin cancer [4].

Pomegranate (*Punica granatum,* Punicaceae) contains anthocyanins and hydrolysable tannins and possesses strong antioxidant and anti-tumor-promoting properties [2,5]. Various scientists have also reported that *P. granatum* constituents are useful against UVB-induced damage to human skin [6,7]. By the bioassay-guided isolation, ellagic acid has been considered as the main active compound of the extract. The phenolic nature of ellagic acid makes itself a powerful antioxidant [8]. It has been observed in various studies that ellagic acid has very short half life if taken orally or intravenously. To overcome this difficulty our approach is to entrap the extract of *P. granatum* consisting of ellagic acid in the nanotransfersomes and incorporate into the cream for the topical administration.

Novel vesicular delivery systems improve the solubility, permeability, and stability of antioxidants [9]. The application of novel approaches can also improve its efficacy regarding continuous action of herbs on the human body [10]. Specially designed liposomal formulations can deliver drugs within 30 min to the stratum corneum, epidermis, and dermis in significantly higher concentrations than conventional preparations [11]. Cevc and Blume claimed that nanotransfersomes, being

ultradeformable (up to 10 times that of liposomes), could squeeze through pores in stratum corneum (less than one-tenth the vesicles diameter) and could penetrate intact skin due to the natural transepidermal water activity gradient [12]. In addition they provide valuable raw material for the regeneration of skin by replenishing lipid molecules and moisture. Lipids are well hydrated and, even in the absence of active ingredients, humidify the skin. Phospholipids used for the preparation of formulation are the safest, mildest substances which allow the penetration of the plant actives into the deeper layers of the epidermis and avoid the use of solvents [13].

Enzymes which convert reactive oxygen species (ROS) to harmless water and molecular oxygen protect skin from ROS-induced damages. The levels of these major endogenous anti-oxidant enzymes, superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase are shown to decrease after a single and repeated exposure to radiation in mice and pig and also in aged and photoaged skin of human [14]. The level of catalase in epidermis is much higher than in the dermis, and decreases after a single UVB and UVA exposure, recovering 3-4 weeks after exposure [4].

In our previous work we had prepared cream formulations of *P. granatum* extract and compared with that of creams produced from *C. longa* extract. The produced creams produced photoprotective effect which was assessed by improvement in skin hydration properties [5]. The main objective of the present study is to prepare and evaluate cream formulations with nanotransfersomes loaded with *P. granatum* extract and to investigate the effect on skin, *in vivo*, by assessing the change in biochemical components and skin hydration. The herbal extract loaded nanovesicles incorporated in the cream will be compared with plain extract loaded creams and base cream. Thus, the results will be useful in designing specific nanovesicular formulations for cosmetic and dermatological application with the aim to equilibrate the deleterious effects of photo carcinogenesis.

Materials and Methods

Materials

The dried seeds of *Punica granatum* were purchased from local herbal distributor and were authentified with the help of herbarium of the Pharmacognosy department of University Institute of Pharmacy, Pt. Ravishankar Shukla University. Alcoholic extraction was done taking ethyl alcohol (90%) of Bengal Chemicals and pharmaceuticals Ltd. Kolkata, India. Soya phosphatidylcholine and sodium deoxycholate were purchased from Himedia Laboratories, Mumbai (India), and Ellagic acid (for standardization of extract) from Sigma Chemicals (St Louis, MO). All the chemicals and reagents used in the study were of analytical grade. Double distilled water was used for all experiments.

Methods

Preparation and phytochemical evaluation of P. granatum extract

Extraction of dried seeds of *P. granatum* seeds was done by a continual hot extraction method, using 90% v/v ethyl alcohol [5]. Various phytochemical tests for alkaloids, carbohydrates, flavanoids, steroids, volatile oils, tannins, phenolic compounds, proteins and amino acids, saponins, aromatic acids, triterpenoids, gum and mucilage were performed for the extract [15]. The antioxidant activity of the extract was assessed by reducing power estimation method in which the antioxidant activity of the extract was compared with that of standard (ascorbic acid) [16]. Total polyphenol content was measured using the Folin-Ciocalteu colorimetric method taking gallic acid as standard [17,5].

Preparation of nanotransfersomes

The nanotransfersomes (T1 to T5) were prepared by modified lipid film hydration technique using rotary evaporator (EVATOR, model no. EV11). Firstly soya lecithin and edge activator (sodium deoxycholate) (85:15% w/w) were dissolved in (2-5 ml) chloroform. The organic solvent was removed by evaporation at temperature 43±5 ^{0} C. A thin lipid film was formed inside the flask wall with rotation. The thin film was kept overnight for complete evaporation of solvent. The film was then hydrated with distilled water containing alcoholic *P. granatum* extract (0.5 to 2%) with gentle shaking for 15 min at 43±5 ^{0} C temperature. The nanotransfersome dispersions were further hydrated up to one hour at 2-8 ^{0} C [18]. The stabilized vesicles with high entrapment efficiency and lower polydispersity index were selected for the preparation of transfersome incorporated cream formulations.

Preparation of cream formulations

Initially base cream (coded BC) was prepared by using a phase inversion technique [19]. The oily constituents used were cetyl alcohol, stearic acid, olive oil, jojoba oil, tea tree oil, polysorbitan monooleate (Span 60), polysorbitan monostearate (Tween 80), lemon grass oil, lavender oil and the aqueous phase comprised of double distilled water, propylene glycol and glycerine [20]. For extract loaded cream formulations (PC1, PC2, and PC3), 1.0 %, 1.5 % and 2 % of extract was added just before adding aqueous phase [5]. While preparing vesicular system loaded cream formulations, the 20 % of aqueous phase of base cream was replaced with the nanotransfersomes. Care was taken that the nanotransfersomes were added when the temperature was reduced to 30ºC so that they could withstand the temperature.[21] Coding TC_0 is for cream having empty nanotransfersomes; PTC_1, PTC_2 and PTC_3 are cream formulations with 1.0%, 1.5% and 2% *P. granatum* extract loaded nanotransfersomes.

Characterization of vesicular formulations

Entrapment efficiency determination

The entrapment efficiency of nanotransfersomes was measured by the ultra centrifugation method. The prepared

nanotransfersomes were placed in centrifugation tube and centrifuged at 14,000 rpm for 30 min. The calculation of the entrapment efficiency of the *P. granatum* extract was achieved by measuring the ellagic acid content which is the major constituent of the extract. The ellagic acid present in *P. granatum* extract was assayed both in the sediment and in the supernatant. The entrapment efficiency was calculated from the relationship [(T-C)]/T x 100 where T is the total amount of ellagic acid that is detected both in the supernatant and sediment, and C is the amount of ellagic acid detected only in the supernatant [22]. The entrapped ellagic acid was determined by High performance liquid Chromatography.

Ellagic acid estimation

The ellagic acid was measured using acetonitrile and ethyl acetate (70:30) as a mobile phase with flow rate 1.2 ml/min, a column temperature of 30 ºC and an injection volume of 20µl. The standard curve of ellagic acid was prepared in the concentrations 5-50 µg/ml and the area under the curve was determined at 255 nm wavelength [23]. Similarly the appropriate aliquot of ethanolic solution of *P.granatum* extract was prepared and 20µl of it was injected and ellagic acid content was determined by the standard curve of pure ellagic acid.

Vesicular size determination

The z-average diameters of nanotransfersomes were determined by Dynamic light scattering (DLS) method using a Malvern 4700 (Malvern Ltd., Malvern, UK) with a 25 mW He–Ne laser and the Auto measure (version 3.2) software. The samples were diluted in order to avoid multiple scattering. As a measure of the particle size distribution, the polydispersity index was calculated which ranges from 0.0 (monodisperse) to 1.0 (very heterogeneous). The measurements were performed at 27ºC at an angle of 90º between laser and detector. For each vesicle composition, four suspensions were measured in triplicate by DLS [24].

Electron microscopy of vesicles

Formulations were visualized by transmission electron microscopy (TEM) (Morgagni 268-D) at an accelerating voltage of 100 kV. The samples were photographed after preparing grid negatively stained by 1% w/v phosphotungstic acid [24]. TEM was performed for nanotransfersomes alone as well as after incorporation into creams to confirm the presence of vesicles after the preparation of cream too.

Stability Studies of Vesicles

To ensure the stability of prepared novel vesicular systems, the stability testing was performed according to the ICH guidelines for new product (ICH Q1AR2 C) in which all the vesicular formulations were kept in amber colored glass vials and stored at normal conditions (25±2º C, 60±5% Relative Humidity) and at accelerated conditions (40±2º C, 75±5% Relative Humidity) for 6 months and change in active constituent concentration was observed after 1 month, 3 months and 6 months using stability Chamber (CHM-6S, Remi Instruments, Ltd.).

Physicochemical evaluations of cream formulations

Several physicochemical parameters were measured for each prepared cream formulation according to the Indian Standard Bureau methods. These physicochemical parameters provided information regarding formula stability and skin compatibility. pH, Non volatile (%), Saponification value, Acid value, Fatty Concentration (% w/w) , Spreadability, (%) Layer thickness (µm) , Ash exam, Viscosity (Cps), Microbial count (CFU g^{-1}) and Erythemal score were determined [5,25]. All evaluations were carried out in triplicate presented as ± standard deviation.

In vitro sun protection factor determination

The *in vitro technique* involves measuring the spectral transmittance at UV wavelengths from 280 nm to 400 nm. SPF was determined for pure herbal extract, base cream, creams having only *P.granatum* extract (0.5-2%), Plain transfersomal creams and *P.granatum* extract loaded transfersomal creams. For all type of formulations the aliquots of 200 µg/ml were prepared. The aliquots prepared were scanned between 290-320 nm and obtained absorbance values were multiplied with the respective EE (λ) values. Then their summation was taken and multiplied with the correction factor (10) to produce SPF values [26].

The observed absorbance values at 5 nm intervals were calculated using formula as follows:

$$SPF_{spectrophootometric} = CF \times \sum_{290}^{320} EE\,(\lambda) \times I\,(\lambda) \times Abs\,(\lambda)$$

Where CF = Correction Factor (10), EE (λ) = Erythemogenic effect of radiation with wavelengthλ, I (λ) = intensity of solar light of wavelength λ, Abs (λ) = Spectrophotometric absorbance values at wavelength (λ). The values of EE (λ) x I (λ) are constants.

Evaluation of anti-photoaging effect of vesicular systems

Animal Housing

Albino mice (male/female) (Wistar strain) of body weight 180 ± 20 g and age approximately 8 weeks were used for the present study. They were kept in well-ventilated area of the period of experiment till thirty days and were housed in polypropylene cages at standard animal house conditions temperature (25±2ºC), relative humidity (60±5%) and 12 h dark/ light cycle. Animals were fed with standard laboratory diet and drinking water ad libitum throughout the experiment [27, 28] . They were maintained as per CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) regulations and the studies were

performed after due permission by IAEC (Institutional Animal Ethics Committee) of the PBRI Institute, Bhopal.

Animal grouping

Sixty animals were divided into ten groups consisting of six animals in each group. A demarcated area of approximately 2 cm^2 on the dorsal surface of the mice was shaved using a soft hair removing cream. A hair removing cream was preferred over a shaving blade to minimize free radical production due to trauma from the blade. The formulation was applied 1 h before UV exposure, once daily to the animals of respective groups [27,29].

The study pattern was designed according to the following manner:

Group I was Control (without UV and/or formulation treated), group II was UV treated, group III was UV treated after base cream application, group IV, V and VI were UV treated after pretreatment with 1.0%, 1.5% and 2.0 % extract loaded creams, group VII was UV treated plus empty nanotransfersome incorporated cream, group VIII, IX and X were UV treated after pretreatment with 1.0%, 1.5% and 2 % extract loaded nanotransfersomal creams.

UV exposure to animals

Animals were anaesthetized using ether (5 ml) so that the animals don't move and the irradiation is homogeneous. The distance from the lamp to the animals' back was kept constant at 35 cm [30]. In the present experiment an ultraviolet lamp (200–400 nm) was used to induce photo stress on the skin of mice. The irradiance of 3.6 ± 0.4 m W/cm2 was exposed to achieve 5 J/cm2 intensity of exposed light radiation. The time of light exposure was calculated by following formula :

$$T\{Min\} = \frac{\text{Irradiation Dose } (J\,cm^{-2}) \times 1000}{\text{Irradiance } (mW\,cm^{-2}) \times 60}$$

Biochemical estimations

After 30 days of study, animals were sacrificed under light ether anaesthesia and their skins were surgically removed. The excess of fascia and adherent tissues were removed by washing with chilled normal saline solution. Portion of skin tissues was weighed and minced on glass plate over ice bags and homogenized to make colloid. The homogenates were centrifuged at 3,000 rpm for 15 min and supernatant fractions were collected and stored in deep freeze for further estimation of biochemical parameters. To study the UV protecting effects of different formulations, biochemical parameters were estimated by reported procedures, i.e., superoxidedismutase [31], malondialdehyde (MDA) [32] , ascorbic acid [33] , catalase [34] and total protein [35] in each set of experiment.

Skin characterization in vivo

Hydration of the epidermis (stratum corneum) was determined with Corneometer® CM 820, sebum content by Sebumeter ® SM 815 and viscoelastic properties with Cutometer ® MPA 580 of Courage and Khazaka, Germany [36]. 0.2 g of the formulations were applied twice daily at the volar fore arm (for skin hydration and viscoelasticity) and at forehead (for sebum content) of human volunteers (age 25- 30 years) with dry UV irradiated skin; in the morning and evening; measurements were taken thrice after 1, 2, 6 weeks of application. All the procedures were performed at constant room temperature (20±5 º C) and relative humidity (45±5%) to minimize variations. This prospective study was conducted as per ethical guidelines taking written informed consent.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Dunnett's test using Graph Pad Prism 3 (Graph Pad Software, Inc., La Jolla, CA, USA).

Results and Discussion

To prevent and improve photoaging besides the use of products aimed at cell renewal stimulation and skin hydration, the development of formulations that act on the dermis in the stimulation of collagen synthesis and in the enhancement of skin firmness is also very important. Hence the use of antioxidant potential of *P. granatum* phytoconstituents along with high penetration and beneficial effects of lipids of nanotransfersomes was the main approach of this study.

Phytochemical evaluation of *Punica granatum* extract

Punica granatum extract was obtained and preliminary phytochemical screening was done which confirmed the presence of triterpenoids, phenolic compounds, saponin, xanthoprotein, flavonoids, aromatic acid, tannins and reducing sugar and absence of alkaloids and steroids. Antioxidant activity of *Punica granatum* extract determined was 29.26± 1.88 % with respect to ascorbic acid and the synergistic value obtained was 142.58 ± 2.82%. Antioxidant properties of medicinal plants have been shown to be due to high content of phenolic compounds [16, 20]. To support it total phenolic content was assessed in terms of gallic acid equivalent and appreciably high amount (78.94 ± 6.40 mg GAE/g) was obtained [5]. The assessment of total phenolic content and antioxidant properties confirmed the photoprotective nature of the extract.

Entrapment efficiency, vesicular size and structure

The nanotransfersomes loaded formulations (T_1 _T_5) with 0.5, 1, 1.5, 2 and 2.5 % *P. granatum* extract were prepared. As observed from table 1, the high entrapment efficiency (80.5 ± 0.04 to 84.3 ± 0.03) with alcoholic extract was produced which indicates that the amount of extract added was sufficient to maintain equal thermodynamic activities in all formulations. By the

RP-HPLC Chromatogram of standard ellagic acid retention time obtained was 2.537 minutes. With the help of this curve as standard the content of ellagic acid in the *P.granatum* extract was assessed as 18.475±0.234 % w/w. By taking this curve as standard the concentration of ellagic acid in the *P.granatum* extract loaded formulations was determined.

The vesicular size obtained was from 175.8 ± 2.0 to 198.4 ± 1.0 nm range which support the penetration in the deeper layers of skin to produce the desired action of photoprotection. When the extract concentration was elevated from 0.5 to 2.5% w/w, a significant (P< 0.05) increase in nanotransfersome size was observed. The size distribution of extract loaded nanotransfersomes as measured by DLS obtained one narrow peak and low polydispersity index (0.2-0.3) confirming the good quality of formulations. Amongst five formulations on the basis of high entrapment efficiency and lower polydispersity index only three formulations (1.0%, 1.5% and 2%) were selected for further cream formation and coded them PT_1, PT_2 and PT_3.

The photomicrographs of transmission electron microscopy (TEM) (Figure 1a) show vesicular structure of the prepared nanotransfersomes. The photomicrographs were also taken after incorporation of nanotransfersomes in cream to confirm the presence of vesicles which was to authentify the procedure that after incorporating into the creams the vesicles were not disrupted they maintained their vesicular structure. (Figure 1b)

Stability Studies of vesicles

Results of stability studies show that vesicles lost around 1-2 % of the active chemical constituent after one month of storage at normal conditions and 2-3 % of the active chemical constituent after six months of storage as shown in the Table 2 .The loss was marginal in the case of vesicles stored at accelerated conditions. The vesicles lost around 2-3 % of the active chemical constituent after one month of storage at normal conditions and 3-5 % of the active chemical constituent after six months of storage. Hence the prepared vesicular formulations were stable at both normal and accelerated conditions. Thus they were ready for the incorporation into the creams.

Physicochemical evaluations of formulations

Creams were prepared and were optimized for their stability at room temperature for six months. The formulations showed no signs of bleeding at room temperature with no color and pH change which indicated the stability of the base cream formula. As depicted in Table 3, the pH of base cream was 5.83±0.01, of extract loaded creams was from 5.20±0.02 to 5.53±0.02; of transfersomes loaded creams was from 5.41±0.02 to 6.09±0.01. Incorporation of the extract decreased the pH. Hence pH of all the cream formulations was in the skin compatibility range as the pH of the mantle of the skin is 5.5 [38].

The acid value is associated with the free fatty acid and volatile content. The acid value was from 6.73±0.3 to 8.24±0.1 range; saponification value was between 24.0±0.5 to 36.78±0.2; the Non volatile % was in the range 14.91±0.5 to 22.24±0.1 ; Fatty Concentration was 12.20±0.3 % w/w to 15.8±0.9; Spreadability % was between 90±2 to 98±1; Layer thickness was 3.24±0.3 to 6.73±0.3µm ; Viscosity in the range 5660±20 to 5967±60 Cps; Ash exam was found to be between 0.02±1 to 0.08±1; microbial count obtained was between 30±2 to 34±1 CFU g-1 and the erythemal score was 0 for all the formulations. The saponification value of the formulations reflects the presence of free esters, which may influence the formula stability, pH and cleansing properties. During storage and handling cosmetic formulations, viscosity affects the formulation's acceptability and the viscosity of all formulations was between 5000 and 6000 cps (Table3) which was highly desirable. The microbial count for all the formulations were low and were within the lower limit as given by Cosmetics, Toiletries and Perfumery Association (CTFA) for cosmetic and toiletry products (100 cfu g^{-1})[38]. With respect to safety and the irritant test evaluation, all the formulations showed erythema score 0 indicating no irritation (no redness) by visual observation according to COLIPA and BIS guidelines. Lower acid and saponification values, high spreadability, optimum pH and viscosity depicts the formation of stable cream formulations.

In vitro sun protection factor (SPF) determination

The SPF is a quantitative measurement of the effectiveness of a sunscreen formulation. To be effective in preventing sunburn and other skin damage, a sunscreen product should have a wide range of absorbance between 290 and 400 nm. Higher is the value of sun protection factor more is the photo protective activity [30]. As observed from figure 2 for extract loaded creams (PC1,PC2,PC3) and novel extract loaded creams (PTC1, PTC2, PTC3) highly significant (P<0.001) SPF vaues were obtained as compared to base cream. In the cream formulations on increasing the extract concentration SPF values increased. The components of base cream resulted in SPF 2.2±0.6, similarly for empty nanotransfersome loaded creams, SPF value was 3.8±0.2 which show that the lipoidal constituents also enhanced the SPF value. Further addition of extract, multiplied the SPF value and highest was obtained 19.4±0.3 for 2% extract loaded novel nanotransfersomal cream.

The *in vitro* SPF values obtained for different type of extract loaded nanotranfersomal formulations were highly significant which may be due to cumulative effect of lipopolysaccharidal nature of soya lecithin present in nanotransfersomes and phytoconstituents present in the extract of *P. granatum*. This parameter also supports the benefits of the prepared extract loaded nanotransfersomal creams as photoprotectives.

Evaluation of anti-photoaging effect of vesicular systems

Biochemical estimations

As observed from table 4, when compared to control, superoxide dismutase, ascorbic acid, catalase and total protein content decreased while malondialdehyde increased when the ultraviolet radiations were exposed on the skin. Further on application of the prepared formulations before ultraviolet radiations the effect of the radiations was reduced. Superoxide dismutase level was 59.69± 3.16 for control and it decreased to 40.05±3.50 by ultraviolet radiations; pre treatment with prepared formulations showed improvement from 42.45± 2.24 to 56.06±3.25. Malondialdehyde was 1.2±0.14 mmoles/mL for control and it increased to 1.96±0.27 by ultraviolet radiations exposure and reduced from 1.8±0.07 to 1.24± 0.08 by the presence of formulations Level of ascorbic acid was 4.09±0.11 mg/100 mL for control, 1.77±.017 after UV radiations and between 1.88 ± 0.24 to 3.77 ± 0.25 by the pretreatment of formulations. Catalase was 32.68±2.75 for control, 21.99 ±2.84 after UV radiations and between 22.32±1.23 to 34.77 ±2.22 by the pretreatment of formulations. Total protein level was 561.37±3.09 ug mL for control, 479.57±19.72 after UV radiations and between 484.57±8.34 to 539.64 ± 3.64 by the pretreatment of formulations.

The antioxidant enzymes, such as superoxide dismutase, catalase and glutathione peroxidase constitute a mutually supportive team of defence against Reactive Oxygen Species (ROS). The improvement in the level of these antioxidant enzymes by the pretreatment of the formulations supports the photoprotective efficacy of the formulations. The increase in malondialdehyde level in skin tissues suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defence mechanisms to prevent formation of excessive free radicals. Pre treatment with cream formulations significantly reversed these changes. The improvement was observed highly significant ($p<0.01$) with extract loaded nanotransfersomal cream formulations than with plain extract loaded creams ($p<0.05$) as compared to direct ultraviolet radiations treated skin. The increase in extract concentration also enhanced these effects. The efficacy was in the order UV+PTC_3> UV+PTC_2> UV+PTC_1 > UV+PC_3 > UV+PC_2 > UV+PC_1> UV+TC_0> UV+BC. By the pre-treatment with the prepared formulations the reversal of the biochemical changes caused by the ultraviolet radiations towards normal was observed which confirm the radical scavenging ability of the prepared formulations. The results are in congruence with that of Gupta and Dixit who studied the effects with curcumin loaded vesicular systems [28].

Skin characterization in vivo

Significant increases in the water content of stratum corneum readings ($P<0.001$) relative to baseline were observed 1, 2 and 6 weeks after the application of all formulations. (Figure 3) The improvement started after one week period and consistently improved till 6 weeks. After 1 week base cream produced 5.47±1.2 percent improvement in skin hydration while the improvement reached upto 10±2 percent increase with respect to control (baseline) after 6 weeks. Similarly for *P. granatum* extract loaded creams improvement was 6.84±3.0 percent after 1 week and 16 ±1 percent after 6 weeks; while *P. granatum* extract loaded nanotransfersomal creams produced 27.48 ±1.5% increase after 1 week and 32.78±2 percent increase after 6 weeks period.

Significant improvement in skin hydration was observed by the long term study of formulations . However, when these formulations were compared with each other, the water content of the stratum corneum values obtained with the nanotranfersome formulation containing 2% w/w of *P. granatum* extract was higher than others. As proposed by Blichmann et al, lipidisation could be the cause of the prolonged increase in the moisture content on the skin and thus prevented loss of moisture [39] . The other constituents present in formulation like glycerine, propylene glycol, tween, span etc act as humectants which promote the retention of moisture by absorbing moisture from the air and by agents acting as keratin softners within the stratum corneum [40].

The results showed that skin humidity was increased significantly by the application of nanotransfersomes over a period of a week compared without treatment. These observations are in agreement with the findings of Roding and Ghyczy, that the long-term application of liposomes caused an increased hydration of the skin [41]. According to Rawling's et al., best moisturizing technology is combination of humectants, emollients (including lipids), and occlusives in single formulation [42]. The results obtained are best suited with these findings as prepared novel formulations have all these ingredients hence produced significant increase in skin hydration [43].

The application of cream improves skin moisture and lipid content due to which it moves towards normal or oily skin type which was very well classified by Youn et al [44]. When we take nanotransfersomes made up of lipids they too accelerate the properties of cream and thereby higher sebum score obtained.

By the exposure of ultraviolet radiations the level of sebum is reduced and skin becomes dry. For control or base line the sebum score was low (46 ± 1 µg cm^{-2}) but it improved by the application of formulations. Sebum score was raised from 70±5 to 120±4 (µg cm^{-2}) for nanotransfersomes, 60±2 to 83±1 (µg cm^{-2}) for extract loaded creams and from 51±2 to 64±3 (µg cm^{-2}) for base cream in 6 weeks (Figure 4). Long term study of novel formulations produced improvement in skin viscoelasticity, reducing the effect of ultraviolet radiations. The significant improvement shows that there are changes in the cellular level which are very prominent after 6 weeks period. The reason may be due to phospholipid content and the penetration of polyphenols leading to their antioxidant effect. The plain base cream also improved the skin viscoelasticity properties which might be due to combined effect of the constituents present in it.

The improvement in the skin viscoelasticity was found to be increased to 58.69 ±2.3% for *P. granatum* extract loaded nanotransfersomal creams, 13.5 ± 1.2% for *P. granatum* extract loaded creams and 2.07±0.5% as compared to the base cream after 6 weeks (Figure 5). The water content of the stratum corneum and the skin surface lipids form a balance that is

important for the appearance and function of the skin [43]. The water content of the stratum corneum, the skin lipid content and the water-binding substances from the hydro-lipid film of the skin, act together as a barrier to the environment [45]. If this balance is disrupted, a dermatologic condition known as "dry skin" is produced. Severe photoaging also leads to dry skin hence the formulations who maintain balances between both are very beneficial. This signifies the preparation of novel herbal nanotransfersomal creams which will act as photochemopreventives by improving skin hydration, lipid content and viscoelasticity which are reduced as a result of photoaging.

Conclusions

As reactive oxygen species generated by ultraviolet radiations damage antioxidative system of skin the use of antioxidant phytoconstituents in topical formulations is a very promising approach. The effects of these herbal constituents are enhanced by the use of nanotransfersomes. In this study the photoprotective nature of the *P. granatum* extract was confirmed and development of cream incorporated with extract loaded nanotransfersomes was successfully done. The improvement in skin properties and biochemical studies confirm that this approach could be used for combating the deleterious effects of ultraviolet radiations which causes photoaging. The efficacy was in the order *P. granatum* extract loaded nanotransfersomal creams > *P. granatum* extract loaded creams > Plain nanotransfersomal cream > Base cream. Better penetration and smaller size of vesicles with antioxidant potential of the phytochemicals of *P. granatum* extract could be the reason for improved skin properties. The application of herbal extract loaded nanovesicular creams could be utilized for the prevention from ultraviolet radiations generated premature aging.

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Table 1: Vesicular size, entrapment efficiency and polydispersity index of size distribution of nanotransfersomes

Formulation Code	Extract concentration % w/w	Entrapment Efficiency %	*z*-average mean (nm) ±S.D	Polydispersity index
T_0	-	-	175.8 ± 2.0	0.256± 0.012
T_1	0.5	80.5 ± 0.04	184.3 ± 2.3	0.205± 0.003
T_2	1	82.8 ± 0.01	187.6 ± 1.2	0.212± 0.023
T_3	1.5	82.2 ± 0.02	190.2 ± 3.0	0.293± 0.014
T_4	2	84.3 ± 0.03	196.2 ± 2.0	0.204± 0.013
T_5	2.5	81.4 ± 0.01	198.4 ± 1.0	0.343± 0.033

Table 2: Stability testing of *P.granatum* extract loaded nanotransfersomes

FC	Percent AC at Normal conditions (25±2ºC, 60±5% Relative Humidity)			Percent AC at Accelerated conditions (40±2º C, 75±5% Relative Humidity)		
	1 Month	**3 Months**	**6 Months**	**1Month**	**3 Months**	**6 Months**
PT_1	99.5 ± 1.1	99.4 ± 1.1	98.0 ± 2.5	98.3 ± 1.4	97.3 ± 1.3	96.1 ± 1.4
PT_2	99.2 ± 1.5	98.8 ± 1.3	98.2 ± 1.1	98.2 ± 1.6	97.1 ± 2.0	96.3 ± 2.5
PT_3	99.6± 1.4	98.7± 1.2	98.1± 1.2	98.9± 1.1	97.2± 1.5	96.4± 2.0

SD is standard deviation, measurements are taken thrice (n = 3), FC: Formulation Code ; AC: Active constituent content

Table 3: Physicochemical evaluation parameters of cream formulations

Formulation Code	pH	Non volatile (%)	Saponification value	Acid value	Fatty Concentration (% w/w)	Spreadability (%)	Layer thickness (µm)	Ash exam	Viscosity (Cps)	Microbial count (CFU g^{-1})	Erythemal score
BC	5.83±0.01	14.91±0.5	24.0±0.5	6.73±0.3	15.8±0.9	90±2	6.73±0.3	0.06±1	5967±60	30±2	0
PC_1	5.53±0.02	22.24±0.1	24.20±0.2	6.95±0.5	13.23±0.4	93±2	4.24±0.4	0.02±2	5900±10	33±2	0
PC_2	5.32±0.02	20.26±0.4	24.34±0.2	7.21±0.4	12.26±0.2	92±1	3.67±0.2	0.03±2	5800±10	34±1	0
PC3	5.20±0.02	19.82±0.3	28.10±0.4	8.68±0.5	12.20±0.3	93±3	3.24±0.3	0.02±1	5725± 15	35±2	0
TC_0	6.09±0.01	19.50±0.4	33.12±0.3	9.51±0.2	14.3±0.4	95 ±2	6.14±0.2	0.03±1	5940±10	31±1	0
PTC1	5.86±0.02	19.21±0.2	34.11±0.2	6.62±0.3	15.20±0.4	97±2	5.64±0.1	0.05±2	5750± 40	31±3	0
PTC2	5.73±0.01	18.01±0.4	35.52±0.4	7.75±0.2	13.20±0.3	98±1	5.32±0.2	0.08±1	5660±20	32±1	0
PTC3	5.41±0.02	16.66±0.2	36.78±0.2	8.24±0.1	12.80±0.4	97±2	4.24±0.3	0.02±4	5650±30	33±1	0

All the values are represented as mean ± SD (n = 3), P < 0.001 in the column
BC= Base cream, PC_1= Cream with 1.0% *P. granatum* extract, PC_2= Cream with 1.5% *P. granatum* extract, PC_3= Cream with 2% *P. granatum* extract, TC_0=Cream with empty nanotranfersomes, PTC_1= Cream with 1.0 % *P. granatum* extract loaded nanotransfersomes, PTC_2= Cream with 1.5 % *P. granatum* extract loaded nanotransfersomes, PTC_3= Cream with 2% *P. granatum* extract loaded nanotransfersomes

Table 4: Effect of cream formulations on Biochemical parameters

Groups	Group details	Superoxide dismutase (Percentage inhibition of NBT reduction)	Malondialdehyde mmoles/mL	Ascorbic Acid mg/100 mL	Catalase (m moles of H_20_2 utilized l min./mg)	Total protein (ug mL)
I	**Control**	59.69± 3.16	1.2±0.14	4.09±0.11	32.68±2.75	561.37±3.09
II	**UV treated**	40.05±3.50	1.96±0.27	1.77±.017	21.99 ±2.84	479.57±19.72
III	**UV+BC**	42.45± 2.24	1.8±0.07	1.88 ± 0.24	22.32±1.23	484.57±8.34
IV	**UV+TC_0**	44.74 ± 2.06	1.75±0.03	2.04 ± 0.12	23.41±1.50	490.14±5.43
V	**UV+PC1**	45.86 ± 2.32	1.70±0.02	2.12 ± 0.10	24.43±1.69	499.14±10.96
VI	**UV+PC2**	47.83 ± 4.38	1.68±0.05	2.28 ± 0.15	25.82±2.32	506.14 ± 2.02
VII	**UV+PC3**	49.04±1.22	1.62 ± 0.19	2.41 ± 0.13	27.43 ±2.06	509.13 ± 1.68
VIII	**UV+PTC1**	52.67±2.04	1. 43 ± 0.15	2.62 ± 0.20	30.45 ±2.12	518.23 ± 3.04
IX	**UV+PTC2**	54.46±2.32	1.35± 0.23	2.98 ± 0.13	32.43 ±2.04	530.41 ± 4.52
X	**UV+PTC3**	56.06±3.25	1.24± 0.08	3.77 ± 0.25	34.77 ±2.22	539.64 ± 3.64

All the values are represented as mean ± SD (n = 3) P < 0.05 in the column; UV stands for ultraviolet radiations

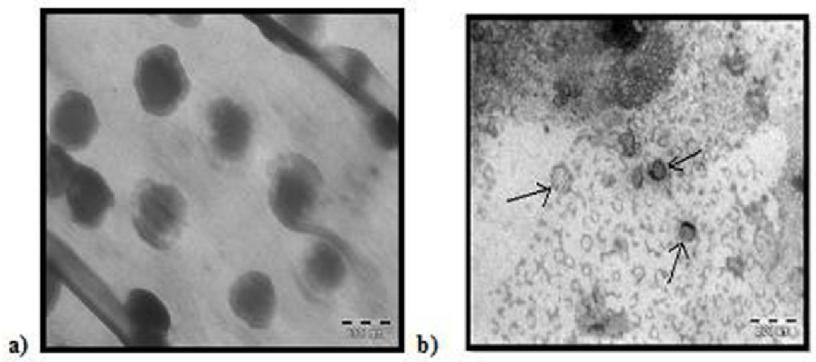

Figure 1: a) TEM of nanotransfersomes at 16,000 X b) TEM of transfersomal cream at 16,000 X

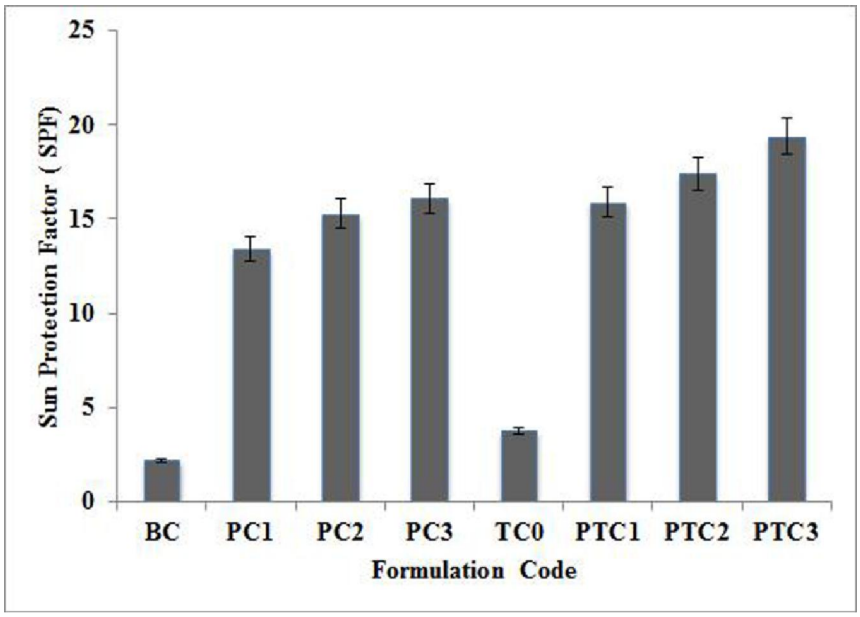

Figure 2: ***In vitro*** **Sun protection factor (SPF) of formulations.** For extract loaded creams (PC_1,PC_2,PC_3) and novel extract loaded creams(PTC_1, PTC_2, PTC_3) highly significant ($P<0.001$) SPF vaues were obtained as compared to base cream, while for empty nanotransfersomes loaded creams non significant ($P>0.05$) SPF value obtained.

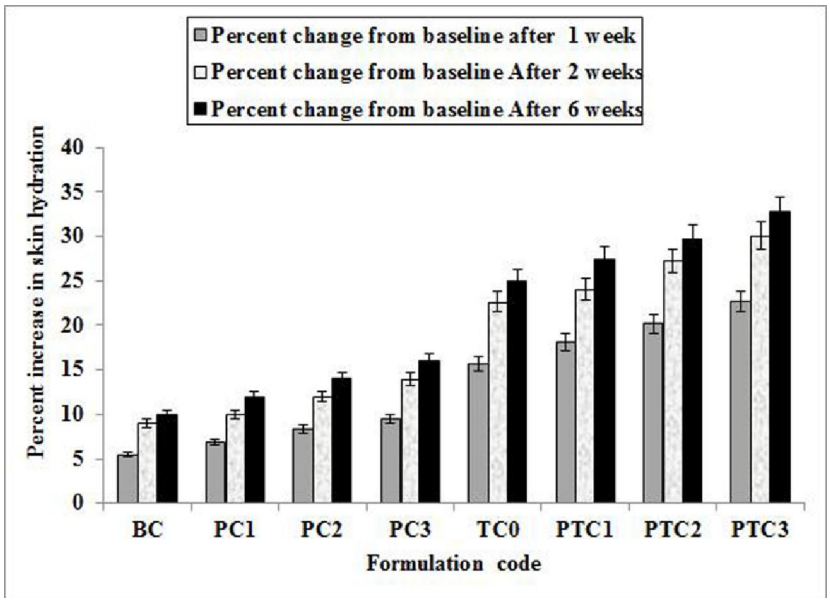

Figure 3: Percent change in skin hydration after formulation application with respect to control or base line. **(n=3), P<0.001 for novel nanovesicular creams while P<0.05 for conventional cream formulations with respect to base line or control**

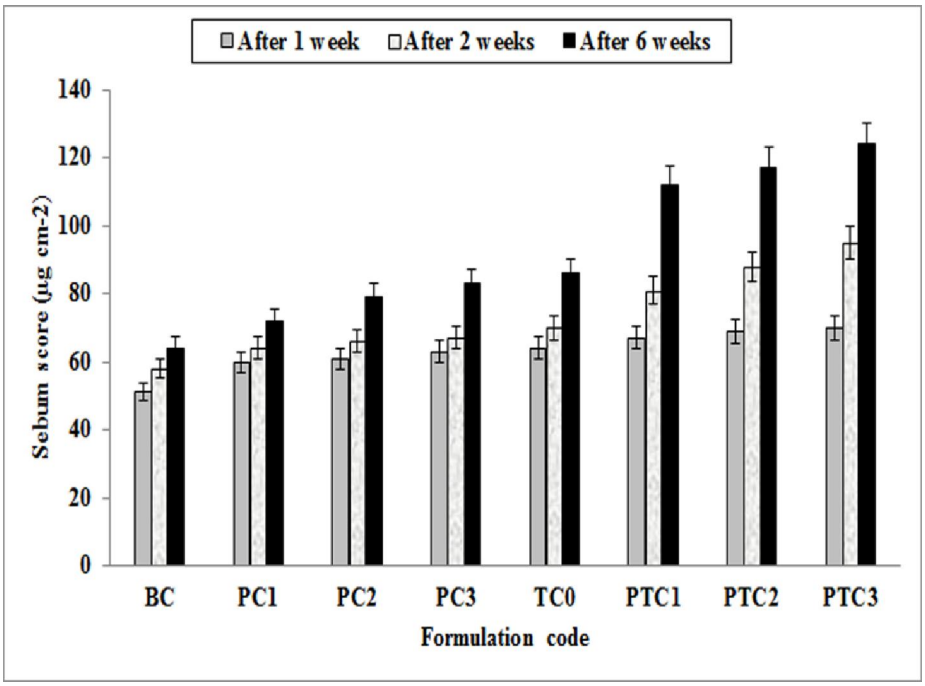

Figure 4: Effect of prepared formulations on skin sebum content
(n=3), P<0.001 for novel nanovesicular creams while P<0.05 for conventional cream formulations with respect to base line or control

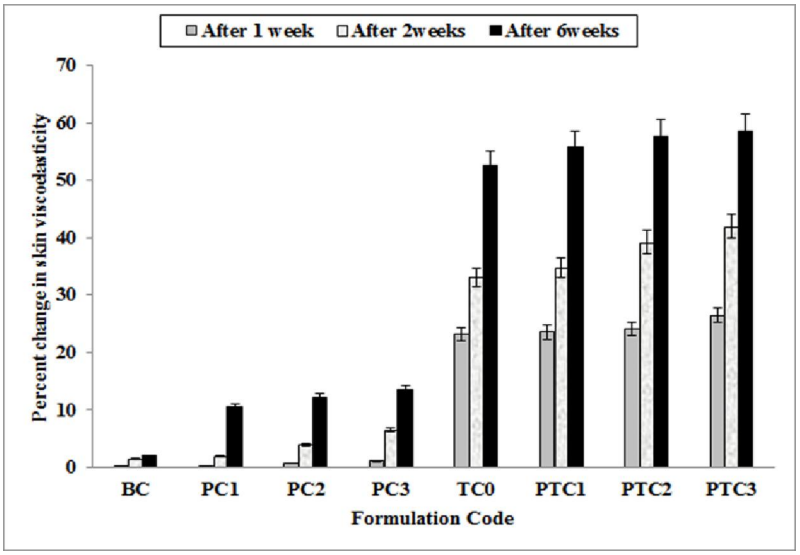

Figure 5: Percent change in skin viscoelasticity after formulation application
(n=3), P<0.001 for novel nanovesicular creams while P<0.05 for conventional cream formulations with respect to base line or control